

# Systemic Correction of a Fatty Acid Oxidation Defect by Intramuscular Injection of a Recombinant Adeno-Associated Virus Vector

THOMAS J. CONLON,<sup>1</sup> GLENN WALTER,<sup>2</sup> RENIUS OWEN,<sup>1</sup> TRAVIS COSSETTE,<sup>1</sup> KIRSTEN ERGER,<sup>1</sup>  
GREG GUTIERREZ,<sup>2</sup> ERIC GOETZMAN,<sup>3</sup> DIETRICH MATERN,<sup>4</sup> JERRY VOCKLEY,<sup>3</sup>  
and TERENCE R. FLOTTE<sup>1</sup>

## ABSTRACT

Mitochondrial  $\beta$ -oxidation of fatty acids is required to meet physiologic energy requirements during illness and periods of fasting or physiologic stress, and is most active in liver and striated muscle. Acyl-CoA dehydrogenases of varying chain-length specificities represent the first step in the mitochondria for each round of  $\beta$ -oxidation, each of which removes two-carbon units as acetyl-CoA for entry into the tricarboxylic acid cycle. We have used recombinant adeno-associated virus (rAAV) vectors expressing short-chain acyl-CoA dehydrogenase (SCAD) to correct the accumulation of fatty acyl-CoA intermediates in deficient cell lines. The rAAV-SCAD vector was then packaged into either rAAV serotype 1 or 2 capsids and injected intramuscularly into SCAD-deficient mice. A systemic effect was observed as judged by restoration of circulating butyryl-carnitine levels to normal. Total lipid content at the injection site was also decreased as demonstrated by noninvasive magnetic resonance spectroscopy (MRS). SCAD enzyme activity in the injected muscle was found at necropsy to be above the normal control mouse level. This study is the first to demonstrate the systemic correction of a fatty acid oxidation disorder with rAAV and the utility of MRS as a noninvasive method to monitor SCAD correction after *in vivo* gene therapy.

## OVERVIEW SUMMARY

Fatty acid oxidation is a critical component in the metabolism of fatty acids as a source of energy. Here we have used pseudotyped AAV1 and AAV2 vectors to overexpress human and murine short-chain acyl-CoA dehydrogenase (SCAD) cDNA *in vitro* and *in vivo* to complement cell lines and in an *in vivo* model containing defective SCAD genes. *In vitro*, we have shown a dose-responsive correction of two human cell lines by monitoring the decrease in the disease-specific metabolite, butyryl-carnitine (C<sub>4</sub>). In addition, the overexpression of SCAD message is demonstrated by semi-quantitative reverse transcription-polymerase chain reaction and Southern blot. *In vivo*, we transduced the muscle

tissue of BALB/c (SCAD<sup>-/-</sup>) mice and demonstrated systemic correction as indicated by a decrease in circulating C<sub>4</sub> and locally by MRS spectroscopy, a novel technique for observing the effects of gene transfer. Together, these data demonstrated the correction of a fatty acid disorder through gene therapeutics.

## INTRODUCTION

GENETIC DISORDERS of mitochondrial fatty acid oxidation (FAO) represent a relatively common class of metabolic disorders. On the basis of newborn screening results, FAO disorders are now known to have a higher incidence than phenylke-

<sup>1</sup>Department of Pediatrics, Powell Gene Therapy Center, University of Florida, Gainesville, FL 32610.

<sup>2</sup>Department of Physiology and Functional Genomics, McKnight Brain Institute, and Powell Gene Therapy Center, University of Florida, Gainesville, FL 32610.

<sup>3</sup>Department of Pediatrics, School of Medicine, University of Pittsburgh, Children's Hospital of Pittsburgh, Pittsburgh, PA.

<sup>4</sup>Biochemical Genetics Laboratory, Division of Laboratory Genetics, Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN.

tonuria (PKU), exceeding more than 1 in 15,000 newborns (Wilcken *et al.*, 2003; Rinaldo *et al.*, 2004). FAO plays a pivotal role in energy metabolism, providing fuel once glycogen stores are depleted during prolonged fasting or during times of increased energy demands (e.g., infections and physiologic stress) (Rinaldo *et al.*, 2002). After liberation from triglyceride stores in adipose tissue, free fatty acids circulate to the liver and striated muscle. After the fatty acids diffuse (short-chain) or are actively transported (long-chain) across the cellular and mitochondrial membranes, the oxidation of fatty acids occurs within the mitochondrial matrix.  $\beta$ -Oxidation proceeds in a cyclical fashion, resulting in the removal of sequential two-carbon units as acetyl-CoA. The first step of each cycle in the mitochondrial matrix is catalyzed by an acyl-CoA dehydrogenase (ACD). This rate-limiting function is performed by a family of enzymes that differ in their substrate specificity based on the carbon chain length of the acyl-CoA molecule.  $\beta$ -Oxidation takes place primarily in metabolically active tissue such as liver, skeletal and cardiac muscle, and brain. In particular, cardiac muscle may acquire up to 70% of its energy requirements from fatty acids (Neely and Morgan, 1974).

At least four ACDs are involved in mitochondrial  $\beta$ -oxidation: short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and very long-chain acyl-CoA dehydrogenase (VLCAD) (Naito *et al.*, 1989; Indo *et al.*, 1991; Tanaka *et al.*, 1992; Coates, 1995; Yamaguchi, 2002; Gregersen *et al.*, 2004). Each enzyme is encoded in the nuclear genome as a precursor containing a mitochondrial targeting peptide at the amino terminus. After translation in the cytoplasm, the precursor proteins are transported to mitochondria, where they are imported into the matrix. The targeting sequence is then removed, and the subunits are folded and assembled into a final homotetrameric form. Each round of the  $\beta$ -oxidation spiral requires the functioning of four enzymes: the rate-limiting dehydrogenase, an enoyl-CoA hydratase, a 3-hydroxyacyl-CoA dehydrogenase, and a 3-oxoacyl-CoA hydratase. The laboratory diagnosis of FAO disorders is based on investigations of blood and urine, including increased acylcarnitine levels by analysis in plasma or blood spots (Rinaldo and Matern, 2005).

The development of rAAV vectors for transduction of myofibers provides a new set of tools with which to explore innovative treatments of FAO disorders. The concept of directing gene therapy to one or the other site relies on the concept that systemic correction may be possible by a small population of transduced cells, that is, that toxic metabolites can be efficiently imported into and cleared by a subset of cells overexpressing the therapeutic protein. Thus, it is highly desirable to have methods available to monitor both focal and systemic correction. In the current study, rAAV gene therapy for SCAD was directed to the muscle of SCAD-deficient (BALB/c) mice. The SCAD mouse model has a natural deletion within the murine SCAD gene resulting in a phenotype similar to that of the human disease, that is, elevated levels of butyryl-carnitine (C4) on fasting, absence of SCAD enzyme activity, and the buildup of fats within the muscle (Amendt *et al.*, 1992). Effects of gene correction were demonstrated by standard methods: systemically by acylcarnitine analysis and locally by measurement of enzyme activity. In addition, a novel method for noninvasively detecting local correction, based on magnetic resonance spec-

trosopy (MRS), has been validated. This new noninvasive methodology will facilitate further preclinical and clinical studies looking at cross-correction by a gene replacement approach.

## MATERIALS AND METHODS

### Vectors

The cDNA sequence for the coding region of murine SCAD was cloned downstream of the cytomegalovirus (CMV) enhancer/chicken  $\beta$ -actin hybrid promoter (Duan *et al.*, 2003) and packaged into capsids of adeno-associated virus (AAV) serotypes 1 and 2. To do this, reverse transcription followed by polymerase chain reaction (PCR) with primers for the human SCAD gene (5'-gtctgtcgcccatggccgc-3' and 5'-gggctcagctccggtagc-3') and murine SCAD gene (5'-ggtctgtgcccattggctgc-3' and 5'-gctcagctccgatagctccg-3') was performed. Resulting bands were subcloned, using the TOPO TA cloning kit as specified by the manufacturer (Invitrogen, Carlsbad, CA). All clones were completely sequenced to ensure that no PCR errors had been introduced. TA donor clones and parental pCB vectors were digested with *EcoRI*, gel purified, and ligated. In addition, *SmaI* digests were used to confirm retention of both AAV inverted terminal repeats (ITRs). Large quantities of experimental plasmids were grown for packaging in the AAV capsids.

### Electron-transferring flavoprotein reduction assay for SCAD enzyme activity

To characterize the enzyme activity and specificity of murine SCAD, the coding region for the mature portion of the enzyme (minus the mitochondrial targeting peptide) was amplified from precursor cDNA clones. A different 5' SCAD primer (SCAD, 5'-atgttacactgtttaccagtctgtggag-3') was used in conjunction with previously described 3' primers. Amplified sequences were cloned in frame into the pET(21a) expression vector (Novagen; EMD Biosciences, San Diego, CA), and the subsequent clones were sequenced in their entirety. The substrate specificity of the SCAD enzyme isolated from crude bacterial cell extracts was characterized with an anaerobic electron-transferring flavoprotein (ETF) reduction assay as described (Kieweg *et al.*, 1997). Crude cellular extracts from the entire muscle tissue of experimental animals were also examined for SCAD activity with this assay. Briefly, on sacrifice of the animal, the tibialis anterior (TA) muscles were removed and flash frozen in liquid nitrogen. For enzymatic assay, the frozen muscle was covered with 0.2 M Tris-HCl, pH 8.0, with complete protease inhibitor cocktail containing benzene-sulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma, St. Louis, MO) and was then homogenized with a Polytron 600 (Brinkmann Instruments, Westbury, NY) for three 10-sec bursts. The sonicate was centrifuged at 4°C for 20 min and the supernatant was collected. Protein concentration was determined by the Bradford method and ACD activity was measured in 100  $\mu$ g of total protein with 5  $\mu$ M butyryl-CoA as substrate. Activity was calculated as milliunits of activity per milligram of protein and experimental samples were compared with controls for significance by analysis of variance (ANOVA).

### *Producing and purifying rAAV2 vectors*

The University of Florida Powell Gene Therapy Center (Gainesville, FL) produced recombinant adeno-associated virus (rAAV) serotype 1 and 2 vectors for these studies. The technique used for AAV serotype 2 included heparan affinity column chromatography (Conway *et al.*, 1999). Virus production included use of the helper/packaging plasmid pDG, which supplies all the necessary helper functions, as well as *rep* and *cap* in *trans* (Grimm *et al.*, 1998). Vector plasmid pCB-SCAD (622.5  $\mu\text{g}$ ) was cotransfected by calcium phosphate precipitation with 1867  $\mu\text{g}$  of pDG into one Cell Factory (Nalge Nunc International, Rochester, NY) of 70–95% confluent 293 cells. Recombinant AAV was purified by iodixanol centrifugation and hand-packed heparan column purification for serotype 2. Physical titer (genome number) was determined by a dot-blot technique. The infectious titer and extent of wild-type AAV2 contamination were determined by infectious center assay (ICA) (Walz *et al.*, 1998). AAV preparations were desalted and concentrated with centrifuge filter devices with a molecular mass cutoff of 100 kDa. The packaging protocols for the AAV2/1 pseudotype are described by Zolotukhin *et al.* (2002). Viral titers were determined by dot-blot assay and expressed as particles per milliliter: AAV2-pCBhSCAD,  $4.92 \times 10^{12}$ ; AAV2/1-pCBmSCAD,  $1.28 \times 10^{13}$ ; AAV2/pCBmSCAD,  $1.59 \times 10^{12}$ ; and AAV2/pCB-GFP,  $2.48 \times 10^{13}$ . AAV serotype 2 physical particle-to-infectious particle ratios were less than 100:1 in all cases.

### *Animals and intramuscular injection*

Eight-week-old female SCAD-deficient (BALB/c) and female control C57BL/6 mice were purchased from the University of Florida Pathology Core and Jackson Laboratories (Bar Harbor, ME) and handled as approved by the University of Florida Animal Care and Use Committee. When performing intramuscular injections, all animals were anesthetized and maintained with 3% isoflurane. Each mouse was then restrained on a thermally heated surgical table to maintain body temperature. The tibialis anterior (TA) muscle was exposed with a 3-mm incision. AAV vectors in a total volume of 50  $\mu\text{l}$ , or phosphate-buffered saline (PBS), was injected into the TA muscle of SCAD-deficient mice, using a 30-gauge insulin syringe (BD Biosciences Pharmingen, San Diego, CA). The wound was closed with 5-0 Ethicon sutures (Ethicon, Somerville, NJ) and the animal was allowed to recover under heat until righting response was observed. Surgeries lasted approximately 10 min. Of note, the ideal control mouse, BALB/cBy, was available only as an embryonic stock at the time of experimentation. Previous experiments in our laboratory have demonstrated comparable C4 levels in both strains containing an intact SCAD sequence.

### *Fibroblast cultures*

All murine and human control and patient primary fibroblasts were grown in modified Eagle's medium (GIBCO; Invitrogen) at 37°C, 5% CO<sub>2</sub> and supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) (all from Mediatech, Herndon, VA). Before use, cells were transduced with adenovirus serotype 5 (Ad5, at a multiplicity of in-

fection [MOI] of 5) expressing the simian virus 40 (SV40) T-antigen and clonally expanded to increase the growth rate.

### *Fatty acid oxidation in vitro probe assay*

The assay was performed according to established protocols (Rinaldo *et al.*, 2002). Probe medium was prepared by adding 175 mg of defatted bovine serum albumin (BSA), 400 mmol of L-carnitine (Sigma), and 200 mmol of palmitic acid (Sigma) to 50 ml of MEM (GIBCO) and shaking the medium overnight at 37°C. For all experiments,  $5 \times 10^5$  cells were first seeded in a 25-cm<sup>2</sup> flask in triplicate, and allowed to settle and double overnight. For cell-mixing experiments, growth medium was replaced with 3 ml of probe medium. For AAV infections, cells were incubated for 1 hr with Ad5 at an MOI of 5 to provide helper functions and to stimulate transgene expression. Cells were washed with PBS and 3 ml of growth medium was replaced. Recombinant AAV vectors were added at doses of 10 and 100 MOI (infectious units) and removed by washing with PBS after 1 hr. Twenty-four hours postinfection, infected cells were probed with 3 ml of probe medium. For cell-mixing and infection experiments, medium samples were taken 60 hr after the addition of probe medium. After a further 60- to 72-hr incubation at 37°C in 5% CO<sub>2</sub>–95% air, medium was collected and frozen at –80°C until analysis. Acylcarnitines were measured as their methyl esters by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) (API 2000; MDS Sciex, Concord, ON, Canada).

### *Semiquantitative reverse transcription-polymerase chain reaction and Southern blotting*

Total RNA was extracted from cultured cells with an RNeasy kit (Qiagen, Valencia, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with 500 pg of total RNA and 8 pmol of the human SCAD primers described above, using a One-Step RT-PCR kit (Qiagen). Cycling conditions included one 15-min reverse transcription cycle followed by 5 min at 95°C for inactivation. The PCR included 1 min at 90°C, 1 min at 54°C, and 2 min at 72°C for 30 cycles. A limiting cycle number of 30 was used to enable semiquantitative measurements of band intensity. Southern blotting was performed with the Gene Images CDP-Star detection kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. A 300-bp SCAD-specific oligonucleotide was used as a probe.

### *Serum acylcarnitine analysis*

Acylcarnitines in serum were measured as their butyl esters as previously described (Rinaldo and Matern, 2005).

### *High-resolution magnetic resonance spectroscopy*

An 11.1-T Bruker Avance spectrometer proton MRS (Bruker BioSpin, Rheinstetten, Germany) was used to determine fatty tissue infiltration. A custom-made loop gap coil with 1.4-cm inner diameter single tuned to 470.5 MHz was used. Localizer anatomical proton magnetic resonance images (matrix, 128  $\times$  128; echo time [TE], 6.5 msec; repetition time [TR], 2500 msec; echo train, 4) were acquired in three orthogonal directions for the precise localization of a volume of interest (VOI) from which proton MRS would be obtained. Magnetic field homo-

genity (~50 Hz) was based on the localized proton spectra. Water suppression was optimized by chemical shift-selective (CHESS) water suppression. Localized spectra were acquired by point-resolved spatially localized spectroscopy (PRESS) (matrix,  $256 \times 256$ ; TE, 18 msec; TR, 6000 msec; number of excitations [NEX], 128). Data were processed with Xwin-nmr (Bruker BioSpin). A Wilcoxon ranked sum test was used to determine any significant difference from zero.

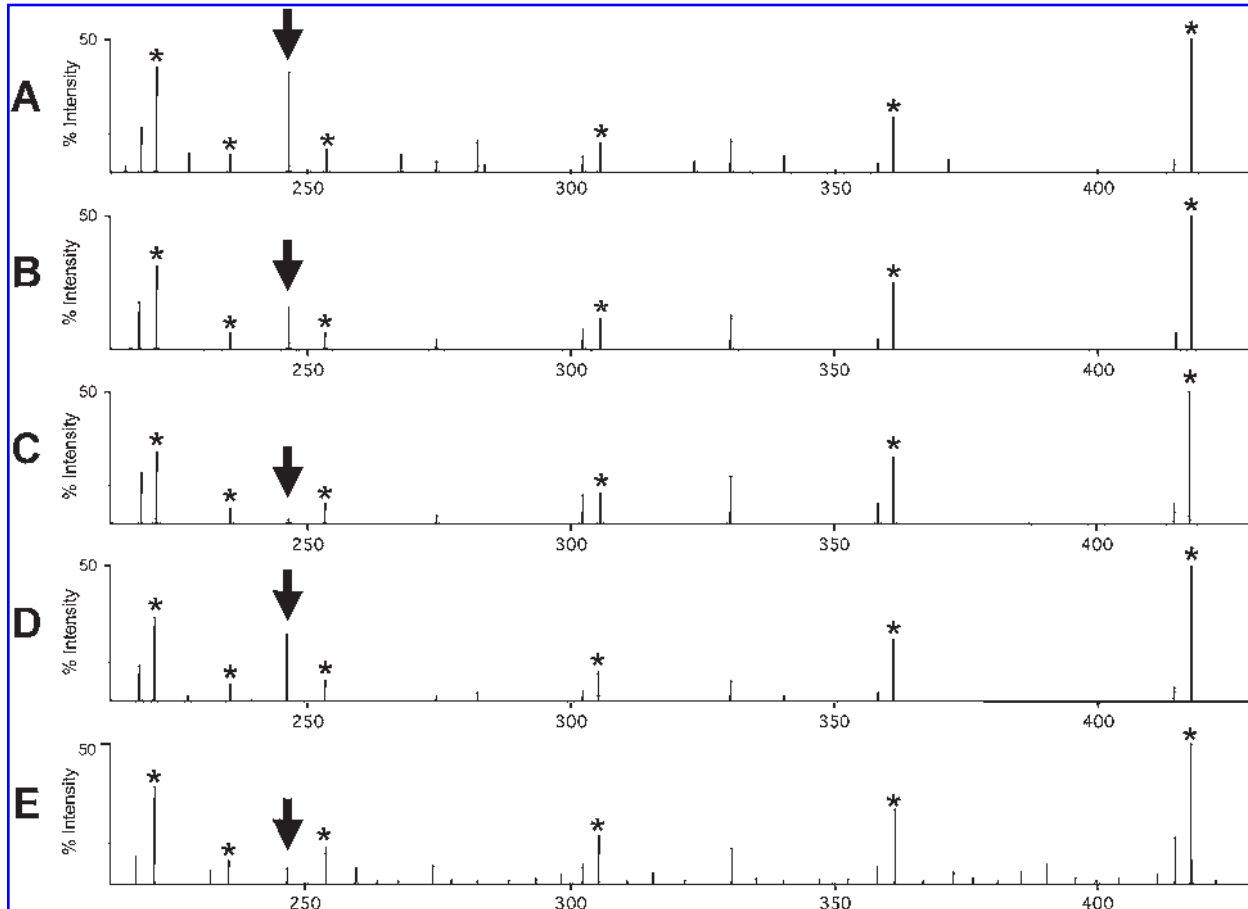
## RESULTS

### Cross-correction of SCAD deficiency in patient cell lines and comparison with cell mixing

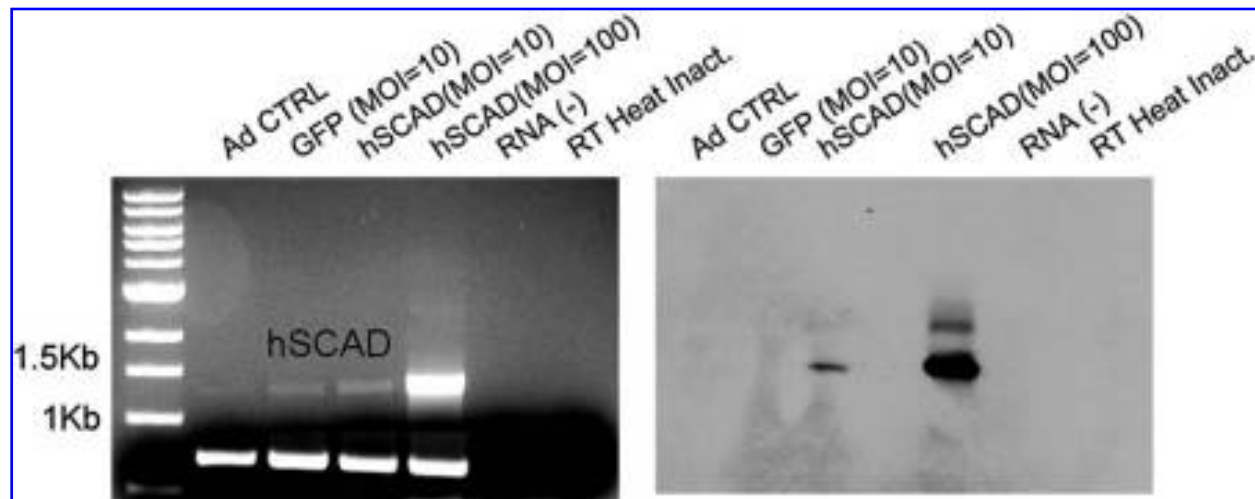
To determine the extent to which correction of a small population of transduced cells can compensate for a lack of SCAD activity and cross-correct SCAD deficiency, we have packaged AAV-2 ITR-based vectors driving expression of human and murine SCAD genes from the constitutively active CMV en-

hancer/chicken  $\beta$ -actin promoter (CB). The resulting human SCAD vector was used *in vitro* to transduce deficient cell lines. Untreated cells, cells infected only with Ad5, and cells infected with AAV2-GFP expressing the green fluorescent protein (GFP) from the CB promoter were used as controls. rAAV/hSCAD was used to treat SCAD-deficient patient cells, at multiplicities of infection of 10 and 100 infectious units (IU) per cell. Sample acylcarnitine profiles from the treated SCAD-deficient human cells showed the butyryl-carnitine (C4) intermediate peak compared with the internal standard peak (\*) decreasing with increasing doses of vector compared with the untransduced control (Fig. 1).

Semiquantitative RT-PCR and Southern blot were performed to confirm overexpression of the SCAD messenger RNA from the CB promoter (Fig. 2). The absence of specific bands in RT-PCR controls with no sample RNA added or heat-inactivated reverse transcriptase demonstrates that bands from treated cells are due solely to endogenous and vector-expressed SCAD RNA transcripts. Low-level mutant SCAD transcripts can also be seen in Ad5 and GFP controls. In addition, a 7-fold increase



**FIG. 1.** AAV2 transduction of human cells *in vitro* reduces detectable butyryl-carnitine (arrow) as measured by tandem mass spectrometry in tissue culture medium after incubation with palmitate and L-carnitine. Representative acylcarnitine profiles (determined 60 hr posttransduction) from cells incubated with adenovirus alone at an MOI of 5 are shown in (A), from cells transduced with AAV2-CBhSCAD at MOIs of 10 and 100 IU/cell, 1 hr after preinfection with adenovirus, in (B) and (C), respectively, untreated SCAD-deficient cells in (D), and normal cells (SCAD<sup>+/+</sup>) in (E). Asterisks (\*) denote deuterated internal standards (H. ten Brink, Klinische Genetica, Amsterdam) (from left to right: d<sub>2</sub>-acetyl-, d<sub>3</sub>-propionyl-, d<sub>7</sub>-butyryl-, d<sub>3</sub>-octanoyl-, d<sub>3</sub>-dodecanoyl-, and d<sub>3</sub>-palmitoylcarnitine).



**FIG. 2.** Semiquantitative RT-PCR and Southern blot detection of human SCAD sequences amplified from mRNA of cells infected with rAAV-hSCAD. SCAD-deficient fibroblasts were allowed to incubate with Ad5 for 1 hr and with AAV vectors for 48 hr and total RNA was isolated. RT-PCR (*left*): Lanes 1 and 2, Ad- and rAAV-GFP-infected controls. The primers amplify a 1.2-kb fragment; lane 3, rAAV-hSCAD-infected cells at an MOI of 10; lane 4, rAAV-hSCAD-infected cells at an MOI of 100. Control reactions without added RNA template or reverse transcriptase are shown in lanes 5 and 6, respectively. A  $\beta$ -actin (700 bp) reaction controlled for the amount of RNA loaded. *Right*: Southern blot of the same samples, using a luminescently labeled probe to the human SCAD gene.

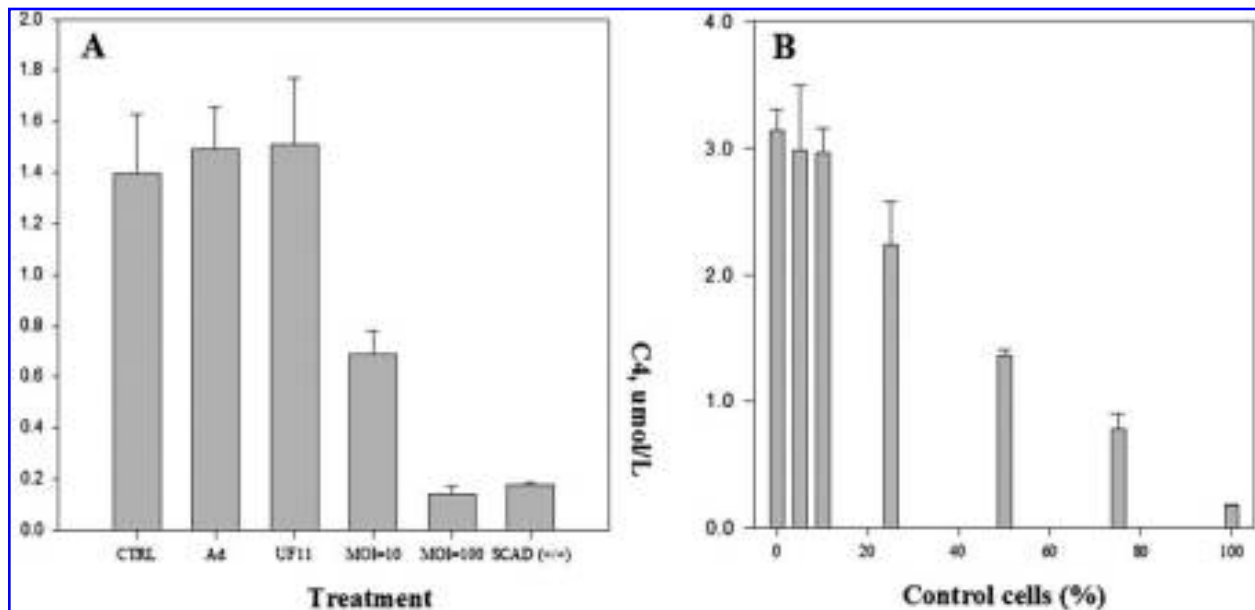
in the intensity of the amplified SCAD-specific band present for an MOI of 10 and a 122-fold induction for an MOI of 100 of SCAD message is observed in relationship to controls when normalized to  $\beta$ -actin reaction loading controls. Importantly, transduction of the deficient fibroblasts led to a reduction in the accumulation of butyryl-carnitine and normalization of butyryl-carnitine between MOIs of 10 and 100 (Fig. 3A).

Translation of gene transfer into gene therapy relies on the concept that cross-correction can occur with a limited number of cells expressing the transgene. One can foresee this concept being achieved with a subset of transduced cells overexpressing the transgene from a highly active promoter. The degree of overexpression needed might be predicted on the basis of physiologic enzyme activities. On the basis of comparable studies (Owen *et al.*, 2000) in fibroblasts, using GFP under the control of the same CB promoter as was used for rAAV-SCAD, we can deduce that a transduction efficiency resulting in less than 30% of the cell population being transduced is achieved at an MOI of 100 on visualization and flow cytometry for GFP-positive cells, although these assays may not allow for detection of low-level expression. Therefore, expressing the SCAD transgene by our vector in no more than 30% of the cell population was required for the observed correction at an MOI of 100 described above, thus reaching the physiologic enzyme activity levels required for correction. In contrast, when SCAD-deficient fibroblasts were mixed with an increasing percentage of normal (i.e., nondeficient) cells, correction to normal control levels was achieved only when 90% or more of the population was expressing SCAD at wild-type levels (Fig. 3B). The true percentage of endogenously expressed SCAD enzyme required may be higher when taking into account that the number of SCAD-deficient cells also decreased as the percentage of normal cells increased. Com-

paring Fig. 3A and B adds additional significance to the use of a highly active promoter.

#### *Muscle transduction diminishes the biochemical and physiologic effects of SCAD deficiency*

Muscle represents an attractive site for therapy, as demonstrated when used for secreted protein disorders in clinical trials (Flotte *et al.*, 2004; Herzog, 2004). The exact mechanism of pathophysiology in  $\beta$ -oxidation defects is unknown. Derangements due to a local cellular energy deficit may lead directly to damage. Alternatively, circulating metabolites related to impaired fatty acid breakdown may be toxic to cells. In the latter case, restoration of normal  $\beta$ -oxidation in skeletal muscle tissue may provide a sink through which harmful metabolites could be reduced. To test this possibility, 8-week-old SCAD-deficient female mice were injected via the TA muscle at one site with PBS, AAV2-CBmSCAD, or AAV2/1-CBmSCAD at a dose of  $9.53 \times 10^{10}$  vector genomes per animal. *In vivo* experiments incorporated the murine SCAD sequence in response to published data indicating a possible immune response to the human gene in mice (Holm *et al.*, 2003). A vector based on the AAV-1 serotype was included because of the documented efficacy of this serotype to transduce muscle tissue as compared with AAV-2 serotype-based vectors (Xiao *et al.*, 1999). The SCAD activity of PBS and vector-induced muscle was measured by electron-transferring flavoprotein (ETF) fluorescence reduction assay, and acylcarnitine levels in transduced mice were quantitated by tandem mass spectrometry. SCAD activity in the muscle of deficient animals was essentially zero (nondetectable), whereas enzyme activity was present in muscle from animals 10 weeks after transduction with either AAV1 or AAV2 SCAD-containing vectors (Table 1).



**FIG. 3.** Recombinant AAV correction of  $\beta$ -oxidation intermediates in cell lines deficient in SCAD. **(A)** Cells were transduced with rAAV vectors at MOIs of 10 and 100 after preinfection with Ad5 at an MOI of 5. The hSCAD and GFP (rAAV-GFP) vectors both used the CB promoter. Forty-eight hours postinfection, medium was collected for acylcarnitine analysis. Butyryl-carnitine (C4) concentrations in two SCAD-deficient human fibroblast cell lines transduced with rAAV-hSCAD were averaged and plotted. The statistical significance of differences between values obtained with the GFP control group and the hSCAD-transduced group (MOI of 100) was determined by unpaired *t* test in **(A)** ( $p < 0.0016$ ). **(B)** Cell mixing of SCAD-deficient and normal human fibroblast cell lines. Cells ( $5 \times 10^6$ ) were plated in triplicate with various percentages of control and SCAD-deficient patient cells. The acylcarnitine accumulation in tissue culture medium was determined by tandem mass spectrometry and butyryl-carnitine values are plotted.

Serum butyryl-carnitine levels were slightly reduced in animals treated with the AAV2-CBmSCAD vector (although the change failed to reach statistical significance), and dropped significantly by nonparametric *t* test ( $p < 0.0149$ ) in animals treated with the AAV-1-based vector (Fig. 4).

Serum acylcarnitine profiles at week 10 postinjection demonstrated systemic and local correction (Fig. 4). A significant difference ( $p < 0.05$ ) between PBS- and AAV1-injected animals demonstrates the ability of injected muscle to compensate for a total lack of activity in the remainder of the animal and to close the gap between SCAD<sup>-/-</sup> and C57BL/6 (SCAD<sup>+/+</sup>) controls by half.

The total lipid concentration in injected TA muscle was examined by MRS scanning and oil red O staining after a 16- to 20-hr fast. Because of the limited presence of fats within the murine TA muscle, detection of fatty acids by staining could not be achieved. However, representative MRS scans (Fig. 5) depict a progressive reduction, SCAD<sup>-/-</sup> > AAV2/mSCAD > AAV1/mSCAD > C57 (SCAD<sup>+/+</sup>), in the lipid peak by comparing the peak height (lipid) with total creatine (TCr = Cr + PCr). The volume of interest was maximized to include as much anterior muscle (primarily the tibialis anterior) as possible, without including noncontractile tissue (i.e., bone). This is shown in Fig. 5A, in which the red outline indicates the outer boundary of where localized spectra were obtained *in vivo*. Group data are shown graphically in Fig. 6. Each subject is plotted as the difference between right and left leg peak values along with the mean for each

group. Of note, individual levels by MRS may vary substantially because of time after last feeding, ambient temperature, activity level, and many other factors that fluctuate during the course of any given day. The use of the contralateral limb as a control reduced variability due to these fluctuating parameters. Whereas the differences between control (C57) and uninjected SCAD mice are negligible, a significant difference ( $p = 0.0029$ ) was observed between treated and untreated TA muscles of mice injected with AAV2/1.

## DISCUSSION

This study demonstrates the feasibility of rAAV-mediated, muscle-directed gene therapy for correction of a fatty acid oxidation disorder. Both local and systemic corrections were demonstrated by standard biochemical genetic techniques (enzyme assay and acylcarnitine analysis, respectively). Furthermore, a noninvasive MRS technique was used to demonstrate local correction of the accumulation of fatty metabolites. These studies provide further insight into the treatment of the more common FAO disorders, medium chain (MCAD) and very long chain (VLCAD), through gene therapy techniques.

Gene replacement using vectors derived from retroviruses has previously been used to correct peroxisomal fatty acid oxidation in adrenoleukodystrophy (ALD) *in vitro* (Cartier *et al.*, 1995) and *in vivo* (Benhamida *et al.*, 2003). ALD deficiency is caused by loss of the ATP-binding transporter, called ALDP,

TABLE 1. SHORT-CHAIN ACYL-Co-A DEHYDROGENASE (SCAD) ENZYME ACTIVITY IN TIBIALIS ANTERIOR MUSCLE OF SCAD-DEFICIENT MICE<sup>a</sup>

Group	Average enzyme specific activity (mu/mg)	SD
PBS (n = 4)	ND	ND
AAV1 (n = 3)	7.2	8.3
AAV2 (n = 3)	19.7	20.6

<sup>a</sup>Tibialis anterior (TA) muscles were extracted on sacrifice at 10 weeks and the electron-transferring flavoprotein (ETF) reduction enzyme assay was performed. No activity (ND, not detected) was observed in PBS-injected animals. Each tissue sample was assayed at least twice. AAV1 and PBS groups were analyzed by ANOVA and found to be nonsignificant ( $p < 0.111$ ).

which is involved in very long-chain fatty acid metabolism in the peroxisome.

Liver-directed gene therapy may also be considered for FAO disorders. Evidence supporting the hypothesis that only liver-directed expression of the SCAD gene may be therapeutic was provided by Kelly and colleagues (1997). This group created transgenic SCAD-deficient (BALB/c) mice, using the liver-specific rat albumin promoter and mouse mini-SCAD gene. In a similar experiment, Holm *et al.* reintroduced the human wild-type SCAD cDNA by a hydrodynamic gene delivery method after injecting a large volume into the tail vein of mice (Holm *et al.*, 2003). An initial decrease in serum butyryl-carnitine levels was observed, but SCAD activity returned to undetectable levels between 2 and 3 weeks after injection. However, liver-directed gene therapy using AAV in humans has been associated with adverse effects including immune responses, hepatocellular damage, and biodistribution to semen. Nonetheless, liver-directed gene therapy for SCAD deficiency would encourage the use of fatty acids in the tissue where  $\beta$ -oxidation naturally predominates.

Current gene therapy phase I trials primarily focus on transducing muscle tissue as a source of secreted therapeutic protein, using AAV2 (Flotte *et al.*, 2004; Herzog, 2004), although therapies using AAV2 directed to the liver are also underway (High, 2003). Treatment of a metabolic disorder typically aims to reduce the concentration of potentially toxic metabolites accumulating as a consequence of a metabolic block. To accomplish this in SCAD deficiency, we hypothesized that restoration of activity in a limited number of muscle cells would allow effective utilization and processing of excess butyryl-CoA accumulated from the rest of the body. The circulating fatty acids would be imported into the subset of corrected cells and processed by the intact  $\beta$ -oxidation pathway, providing a systemic effect. In addition, a local effect would be observed as the accumulation of fatty acids would decrease in primary reservoirs of fats. To evaluate this, we have compared the effectiveness of AAV serotypes 1 and 2 expressing the murine SCAD cDNA in the tibialis anterior (TA) muscle of SCAD-deficient mice to reduce circulating butyryl-CoA levels, while increasing SCAD enzyme activity and decreasing fat accumulation in muscle tissue during fasting. Of note,

when comparing serotypes 1 and 2 in muscle, AAV2/1 vectors had a greater overall effect on reducing circulating butyryl-carnitine, whereas AAV2/2-treated animals produced greater enzyme specific activity. On the basis of other studies (Xiao *et al.*, 1999) it is possible that the distribution of vector throughout the muscle mass may have been broader when using the AAV1-pseudotyped vector; however, insufficient material was available for immunohistochemistry (IHC) to confirm this. If the hypothesis is true, a greater number of cells containing properly mitochondrial-imported protein and enzyme formation could be present in the rAAV1 group and account for the greater overall reduction in lipid within the muscle mass, and this effect is represented by MRS. Meanwhile, the rAAV2 group would be expected to have higher activity per cell in a smaller number of transduced cells. Thus, when normalized to the total protein present the rAAV2 group would have a higher specific activity despite a lesser degree of correction in the whole animal. One limitation of these findings is the lack of a dose-response relationship, which may have clarified the observed difference between serotypes. The MRS imaging method to visualize fat levels represents a novel tool for the study of fatty acid oxidation disorders, allowing noninvasive quantification of total lipids within muscle. A similar approach both to treatment and monitoring has been demonstrated in glycogen storage diseases (Fraitas *et al.*, 2002).

Although preferable regarding human administration and vector spread, potential limitations to intramuscularly directed rAAV-gene transfer for metabolic disease must also be considered. Immune responses to transgene products have been observed under some, but not all, circumstances (Aubert *et al.*, 2003; Louboutin *et al.*, 2004). Immune responses to capsid components occur with regularity and could limit the utility of readministration. Nonetheless, in selected patient populations a single injection could exert a long-lasting effect.

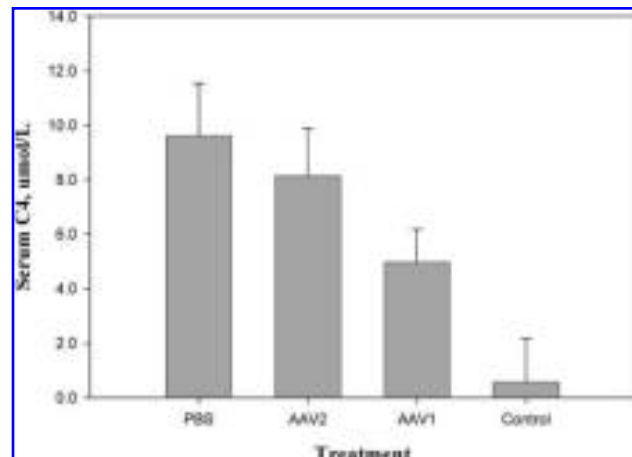
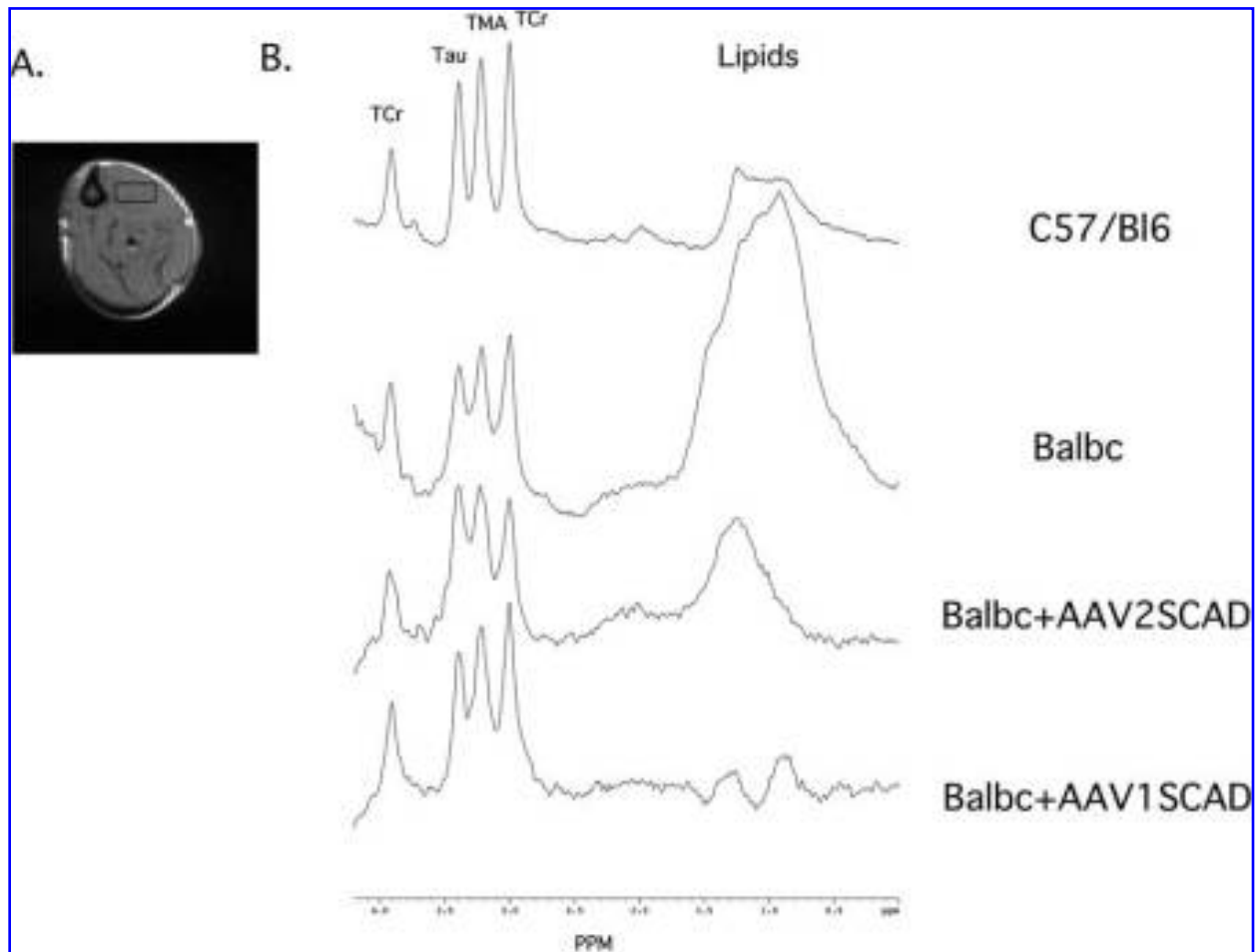
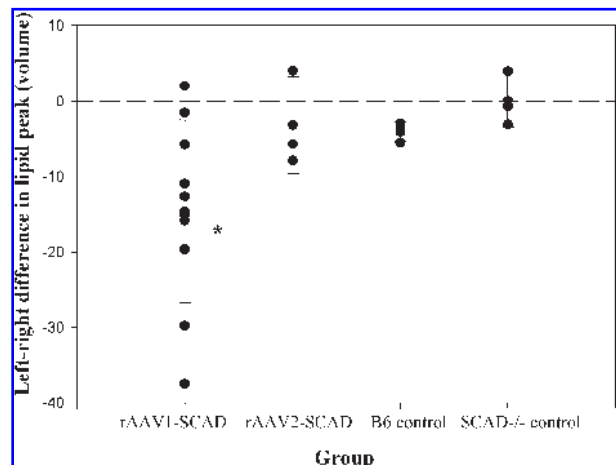


FIG. 4. Serum butyryl-carnitine levels (C4) 10 weeks after intramuscular injection of SCAD-deficient mice with  $9.53 \times 10^{10}$  vector genomes per animal of AAV serotypes 1 ( $n = 8$ ) and 2 ( $n = 3$ ), or PBS ( $n = 5$ ); control, C57BL/6 ( $n = 4$ ). A significant difference is seen between the AAV1-treated cells (\*) and PBS-treated cells as determined by nonparametric  $t$  test ( $p < 0.0149$ ).



**FIG. 5.** Representative MRS proton spectra of rAAV-mSCAD-injected SCAD-deficient mouse tibialis anterior (TA) muscle. (A) Single transaxial MR image of the lower mouse hind limb, used to guide acquisition of the proton MRS spectra. Black box indicates the region from which proton spectra were acquired ( $1 \times 2 \times 3 \text{ mm}^3$ ) and shows where localized spectra were obtained. (B) Ten weeks after intramuscular injection of each animal with  $9.53 \times 10^{10}$  vector genomes of AAV2-pCBmSCAD ( $n = 3$ ) or AAV1-pCBmSCAD ( $n = 8$ ) (control mice, SCAD-deficient BALB/c [ $n = 3$ ] and C57 [ $n = 3$ ]), TA muscles were scanned by proton MRS for lipid volume. Labeled resonances included total lipid (free fatty acids and acyl-CoAs) and total creatine (TCr). The  $^1\text{H}$  spectrum is elevated in SCAD-deficient (BALB/c) untreated muscle versus C57BL/6 control mouse. Intermediate lipid spectra representing a reduction in total lipid can be seen in the AAV2-injected mouse and further reduction may be observed in the AAV1-pCBmSCAD-injected TA muscle. We did not find any differences in image contrast between the various treatment groups. TCr, total creatinine; Tau, taurine; TMA, trimethyl ammonium.

**FIG. 6.** Difference in lipid content of injected and untreated TA muscles from the same animals. One leg of each mouse was treated with  $9.53 \times 10^{10}$  vector genomes of either AAV1 or AAV2 vector carrying the murine SCAD cDNA, and the injected leg and contralateral leg were subjected to MRS scanning for lipid concentration 10 weeks posttreatment. Untreated C57BL/6 (SCAD $^{+/+}$ ) and BALB/c (SCAD $^{-/-}$ ) mice were used as controls. Left leg values were subtracted from right leg values to obtain the difference in lipid concentration. Significance (\*) was obtained between the rAAV1-SCAD-treated group ( $p < 0.0029$ ) as determined by Wilcoxon ranked sum test.



Importantly, the use of MRS will allow monitoring of local transgene effects and correlation to systemic metabolite correction.

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Address reprint requests to:

*Dr. Terence R. Flotte*  
*University of Florida, Box 100296*  
*1600 SW Archer Road*  
*Gainesville, FL 32610*

*E-mail: flotttr@peds.ufl.edu*

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